

## Claims

1. A method for the amplification of nucleic acids comprising the following steps

1) isolating a nucleic acid sample,

2) treating said sample in a manner that differentiates between methylated and un-methylated cytosine bases within said sample,

3) amplifying at least one target sequence, within said treated nucleic acid, by means of enzymatic amplification and a set of primer molecules, wherein said primer molecules are characterized in that

a) each primer molecule sequence reaches a predefined measure of complexity,

b) every combination of any two primer molecules in the set has a melting temperature below a specified threshold temperature,

c) every combination of two primer molecules, under conditions allowing for one or more base mismatches per primer, does not lead to the amplification of an unwanted product when virtually tested using the treated and the untreated sample nucleic acids as template,

and

4) detecting said amplified target nucleic acid.

2. A method according to claim 1 wherein said primer molecules do not contain nucleic acid sequences com-

plementary or identical to nucleic acid sequences of the target sequence which prior to treatment of step 2 contained a 5'-CG-3' site.

- 5     3. A method according to claims 1 and 2 wherein said set is comprised of at least one but not more than 32 primer pairs.
- 10    4. A method according to claims 1 and 2 wherein said set is comprised of at least one but not more than 16 primer pairs.
- 15    5. A method according to claims 1 to 4 wherein the primer molecules are reaching a specified value of linguistic complexity.
- 20    6. A method according to claims 1 to 4 wherein the primer molecules are reaching a specified value of Shannon entropy.
- 25    7. A method according to claims 1 to 6 wherein the nucleic acid sample is isolated from a bodily fluid, a cell culture, a tissue sample or a combination thereof.
- 30    8. A method according to claims 1 to 7 wherein the nucleic acid sample is comprised of plasmid DNA, BACs, YACs or genomic DNA.
- 35    9. A method according to claims 1 to 7 wherein the nucleic acid sample is comprised of human genomic DNA
10. A method according to claims 1 to 9 wherein said sample is treated by means of a solution of a bisulfite, hydrogen sulfite or disulfite.

11. A method according to claims 1 to 10 wherein said primer molecule comprises of at least one nucleotide within the last three nucleotides from the 3' end of the molecule wherein said nucleotide is complementary to a nucleotide of the target sequence that was converted to a different nucleotide by the treatment performed in step 2) of claim 1.

12. A method according to claims 1 to 10 wherein said primer molecule comprises of at least one nucleotide within the last three nucleotides from the 3' end of the molecule wherein said nucleotide is complementary to a nucleotide of the target sequence that was converted to a different nucleotide by bisulfite treatment.

13. A method according to claims 1 to 12 wherein each of said primer molecules is characterized in that the last at least 5 bases at the 3' end of said primer molecule are not complementary to the sequence of any other primer molecule in the set.

14. A method according to claims 1 to 13 wherein the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 20% of the number of nucleotides of the primer molecule.

15. A method according to claims 1 to 13 wherein the number of nucleotides creating one gap, when aligning the primer molecule sequence with the template sequence, allowed for, when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 20% of the number of nucleotides of the primer molecule.

16. A method according to claims 1 to 13 wherein the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 10% of the number of nucleotides of the primer molecule.
17. A method according to claims 1 to 13 wherein the number of nucleotides creating one gap, when aligning the primer molecule sequence with the template sequence, allowed for, when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 10% of the number of nucleotides of the primer molecule.
18. A method according to claims 1 to 13 wherein the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 5% of the number of nucleotides of the primer molecule.
19. A method according to claims 1 to 13 wherein the number of nucleotides creating one gap, when aligning the primer molecule sequence with the template sequence, allowed for, when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than 5% of the number of nucleotides of the primer molecule.
20. A method according to claims 1 to 13 wherein the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is less than seven.
21. A method according to claim 20 wherein the number of mismatches allowed for is less than five.

22. A method according to claim 20 wherein the number of mismatches allowed for is less than three.

23. A method according to claim 20 wherein the number of mismatches allowed for is one.

24. A method according to claims 1 to 13 wherein the number of mismatches allowed for when virtually testing the amplification of unwanted products according to step 3 c) of claim 1 is determined by a pre-specified maximum melting temperature.

25. A method according to claims 1 to 24 wherein said primer molecules are used to amplify nucleic acid sequences that prior to treatment of step 2 comprised of more than eight 5'-CG-3' sites.

26. A method according to claims 1 to 24 wherein said primer molecules are used to amplify nucleic acid sequences that prior to treatment of step 2 comprised of more than six 5'-CG-3' sites.

27. A method according to claims 1 to 24 wherein said primer molecules are used to amplify nucleic acid sequences that prior to treatment of step 2 comprised of more than four 5'-CG-3' sites.

28. A method according to claims 1 to 24 wherein said primer molecules are used to amplify nucleic acid sequences that prior to treatment of step 2 comprised of more than two 5'-CG-3' sites.

29. A method according to claims 1 to 28 wherein the ability of said primer molecules to amplify an unwanted product is tested by means of electronic PCR.

30. A method according to claims 1 to 28 wherein the ability of said primer molecules to amplify an unwanted product is tested by means of electronic PCR, taking as template nucleic acid the coding strand of the treated sample, the non-coding strand of the treated sample and both of the strands of the untreated sample.

31. A method according to claims 1 to 28 wherein the ability of said primer molecules to amplify an unwanted product is tested by means of electronic PCR, taking as template nucleic acid the coding strand of the bisulfite converted human genome, the non-coding strand of the bisulfite converted human genome and both of the strands of the untreated human genome.

32. A method according to claims 1 to 31 wherein said primer molecules are used to amplify nucleic acids which are comprised of at least 50 bp but not more than 2000 bp.

33. A method according to claims 1 to 31 wherein said primer molecules are used to amplify nucleic acids which are comprised of at least 80 bp but not more than 1000 bp.

34. A method according to claims 1 to 33 wherein said primer molecules are comprised of 16 - 50 nucleotides.

35. A method according to claims 1 to 34 wherein said primer molecules do not form dimers with each other.

36. A method according to claims 1 to 35 wherein said primer molecules do not form loops or hairpin structures.

37. A method according to claims 1 to 36 wherein said primer molecules are complementary to target sequences which prior to the treatment performed in step 2) of claim 1 did not contain specified restriction enzyme recognition sites.

38. A method according to claims 1 to 37 wherein said primer molecules amplify regions of the treated nucleic acids which prior to the treatment performed in step 2) of claim 1 did not contain specified restriction enzyme recognition sites.

39. A method for designing primers according to claim 1, comprising the steps of

a) selecting a pool of possible primer pairs per amplificate by means of a standard PCR primer design program using said nucleic acids as template

b) excluding those primer pairs which comprise of a primer that in combination with another primer molecule in the same set exceeds a threshold melting temperature

c) excluding those primer pairs which comprise of a primer that does not reach a specified level of complexity

d) excluding those primer pairs which comprise of a primer that in combination with another primer molecule in the same set, under conditions allowing for one or more base mismatches per primer, amplifies an unwanted product when virtually tested using the treated and the untreated sample nucleic acid as template.

40. A method for designing said primer molecules according to claim 1 and 43, adding the step of

5 e) excluding from the remaining confirmed primer pairs those pairs which in said amplification step do not result in the amplification of the intended product when performing a single PCR experiment.

10 41. A method for designing primers according to claims 39 and 40 , wherein said template nucleic acids are masked for repeats and SNPs before designing said primer molecules and wherein said standard PCR primer design program considers one or more of the following  
15 factors

length of amplificate, length of primer, melting temperature of the primers, dimer formation parameters, loop formation parameters, exclusion of unidentified  
20 or ambiguous nucleotides in the primer sequence, exclusion of restriction enzyme recognition sites.

42. A method according to claims 39 to 41 wherein said  
25 measure of complexity is a measure of linguistic complexity.

43. A method according to claims 39 to 41 wherein said  
30 measure of complexity is a measure of Shannon entropy.

44. A method according to claims 39 to 43 wherein the  
following step is carried out prior to performing  
step d)

35 excluding from the remaining primer pairs those

pairs, which consist of a primer molecule that comprises of at least one CpG site.

- 5 45. A method according to claims 39 to 44 wherein the following step is carried out prior to performing step d)

10 excluding from the remaining primer pairs those pairs, which consist of a primer molecule that does not contain at least one nucleotide within the last three nucleotides from the 3' end of the molecule wherein said nucleotide is complementary to a nucleotide of the target sequence that was converted to a different nucleotide by the treatment performed in  
15 step 2) of claim 1.

- 20 46. A method according to claims 39 to 45 wherein the following step is carried out prior to performing step d)

25 excluding from the remaining primer pairs those pairs, which consist of a primer molecule that contains more than 5 bases at its 3' end that are complementary to any other primer molecules' sequence in the set.

- 30 47. A method according to claim 39 to 46 wherein the following step is carried out prior to performing step d)

35 excluding from the remaining primer pairs those pairs, which amplify a nucleic acid that did not, prior to the treatment in step 2 of claim 1, contain at least two CpG sites

48. A method according to claim 39 to 47 wherein the following step is added before performing step d)

5 excluding from the remaining primer pairs those  
pairs, which comprise of one primer molecule that in  
combination with another primer molecule in the set  
amplifies an unwanted product, when virtually testing  
according to step 3 c) of claim 1 under conditions  
allowing for a number of mismatching nucleotides of  
10 20% of the number of nucleotides of the primer molecule.

49. A method according to claim 39 to 47 wherein the following step is added before performing step d)

15 excluding from the remaining primer pairs those  
pairs, which comprise of one primer molecule that in  
combination with another primer molecule in the set  
amplifies an unwanted product, when virtually testing  
according to step 3 c) of claim 1 under conditions  
20 allowing for a number of nucleotides creating one  
gap, when aligning the primer molecule sequence with  
the template sequence, of up to 20% of the number of  
nucleotides of the primer molecule.

- 25 50. A method according to claim 39 to 47 wherein the  
following step is added before performing step d)

30 excluding from the remaining primer pairs those  
pairs, which comprise of one primer molecule that in  
combination with another primer molecule in the set  
amplifies an unwanted product, when virtually testing  
according to step 3 c) of claim 1 under conditions  
allowing for four or less mismatching base pairs.

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51. A method according to claim 39 to 47 wherein the following step is added before performing step d)

5       excluding from the remaining primer pairs those  
      pairs, which comprise of one primer molecule that in  
      combination with another primer molecule in the set  
      amplifies an unwanted product, when virtually testing  
      according to step 3 c) of claim 1 under conditions  
10       allowing for two or less mismatching base pairs.